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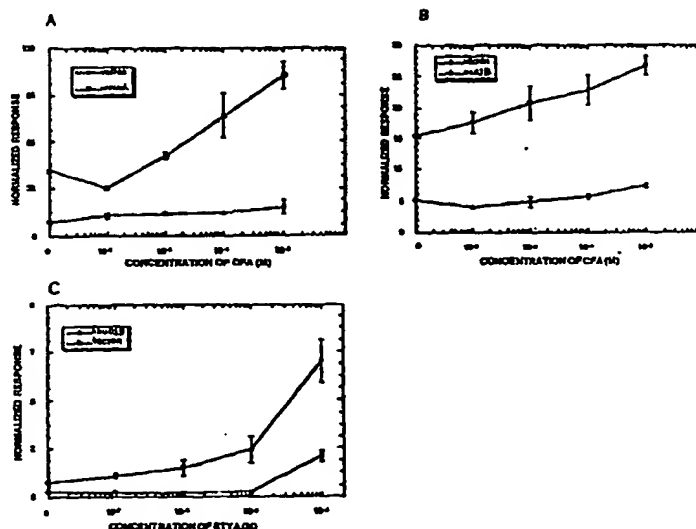
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(54) Title: SCREENING FOR NUC INHIBITORS



(57) Abstract

This invention provides methods for screening for agents useful for treatment of diseases and pathological conditions affected by the level of NUC protein activity. These agents reduce or relieve the repression of PPAR α protein and TR protein transcription activation activity by NUC protein. The selected novel or unique agents can be used to treat hyperlipidemia, hypercholesteremia and hyperlipoproteinemia.

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DESCRIPTION

SCREENING FOR NUC INHIBITORS

Cross Reference to Related Applications

This application is related to U.S. Application Serial No. 08/143,215, titled "Human Peroxisome Proliferator Activated Receptor," filed October 25, 1993, by Mukherjee, which is a continuation-in-part of Application Serial No. 08/141,500, titled "Human Peroxisome Proliferator Activated Receptor," filed October 22, 1993, by Mukherjee; the disclosures of which are incorporated herein by reference.

FIELD OF THE INVENTION

This invention relates to screening for agents active on peroxisome proliferator activated receptor (PPAR) and thyroid hormone receptor (TR) related molecules. This invention also relates to the cloning and sequencing of a new human peroxisome proliferator activated receptor named hNUC1B.

BACKGROUND OF THE INVENTION

Peroxisomes are subcellular organelles found in animals and plants. Peroxisomes contain enzymes for cholesterol and lipid metabolism and respiration.

A variety of chemical agents called peroxisome proliferators induce the proliferation of peroxisomes. Peroxisome proliferators include unsaturated fatty acids, hypolipidemic drugs (Reddy, J. K., and Azarnoff, D. L. (1980) *Nature* 283, 397-398), herbicides, leukotriene antagonists, and plasticizers (for a review, see Green, S., *Biochem. Pharmacol.* 43:393-400, 1992). Hypolipidemic drugs such as clofibrates have been found to lower triglycerides and cholesterol levels in plasma and to be beneficial in the prevention of ischemic heart disease in individuals with elevated levels of cholesterol (Havel, R.J. and Kane, J.P., *Ann. Rev. Pharmac.* 13:287-308, 1973).

Therapeutic use of such drugs, however, is questioned because clofibrates are carcinogens in rats.

There are two hypotheses for peroxisome proliferation. The "lipid overload hypothesis" suggests that an increase in the intracellular concentration of fatty acids is the main stimulus for peroxisome proliferation (Nestel, P. J. (1990) *Ann. Rev. Nutr.* 10, 149-167 and Phillipson, B. E., Rothrock, D. W., Connor, W. E., Harris, W. S., and Illingworth, D. R. (1985) *N. Engl. J. Med.* 312, 1210-1216). Another hypothesis postulates a receptor mediated mechanism. Peroxisome proliferator activated receptors (PPARs) have been isolated and cloned from various species (Isseman, I., and Green, S. (1990) *Nature* 347, 645-650; Dreyer, C., Krey, G., Hansjorg, K., Givel, F., Helftenbein, G., and Wahli, W. (1992) *Cell* 68, 879-887; Gottlicher, M., Widmar, E., Li, Q., and Gustafsson, J. A. (1992) *Proc. Natl. Acad. Sci. USA* 89, 4653-4657; Sher, T., Yi, H. F., McBride, W. O. and Gonzales, F. J. (1993) *Biochemistry* 32, 5598-5604; and Schmidt, A., N. Endo, S. J. Rutledge, R. Vogel, D. Shinar, and G. A. Rodan. (1992) *Mol. Endocrinol.* 6, 1634-1641-8). The ligand for PPARs is still unidentified.

Isseman and Green, *Nature* 347:645-650, 1990, cloned a mouse peroxisome proliferator activated receptor (mPPAR) gene from a mouse liver complementary DNA (cDNA) library. Gottlicher et al., *Proc. Nat. Acad. Sci. USA* 89:4653-4657, 1992, cloned a rat peroxisome proliferator activated receptor (rPPAR) gene from a rat liver cDNA library. PPARs from mouse and rat share 97% homology in amino acid sequence and a particularly well-conserved putative ligand-binding domain. Three members of the Xenopus nuclear hormone receptor superfamily (i.e., XPPAR α , XPPAR β and XPPAR γ) have also been found to be structurally and functionally related to the mPPAR (Dreyer et al., *Cell* 68:879-887, 1992).

Schmidt et al., *Molecular Endocrinology* 6:1634-1641,

1992, cloned a steroid hormone receptor gene, hNUC1, from a human osteosarcoma cell cDNA library. The homology between amino acid sequence of hNUC1 and that of mPPAR is 62%.

5 Sher et al., Biochemistry 32:5598-5604, 1993, cloned a human PPAR gene from a human liver cDNA library. This clone has 85% nucleotide sequence homology and 91% amino acid sequence homology with the mPPAR clone.

Peroxisome proliferator activated receptor (PPAR) is
10 a member of the steroid receptor family. PPAR is divided into several subfamilies based upon their primary sequence homology. Fang et al., Biochem. Biophys. Res. Com. 196:671-677, 1993 discussed XPPAR α , XPPAR β , XPPAR γ , mPPAR and hNUC1.

15 SUMMARY OF THE INVENTION

This invention relates to the cloning, sequencing and expression of a human peroxisome proliferator activated receptor subtype, hNUC1B. hNUC1B differs in amino acid sequence from hNUC1 by one amino acid (i.e.,
20 alanine at position 292).

Applicant has determined that hNUC1B protein represses hPPAR α (hPPAR α , referred to as hPPAR1 in U.S. Application Serial No. 08/143,215, is a subtype of PPAR) and TR protein activity, and that relief from such
25 repression is therapeutically useful.

Thus, the present invention features methods for identifying therapeutic agents that alleviate the repressive effects of NUC protein on PPAR α protein and TR protein activity and for using these agents to treat
30 diseases and pathological conditions affected by the level of NUC protein activity, such as, but not limited to, hyperlipidemia, hypercholesteremia and hyperlipoproteinemia. These methods make it possible to screen large collections of natural, semisynthetic, or synthetic
35 compounds for therapeutic agents that relieve the repression of PPAR α or TR activity by NUC protein.

By "PPAR α protein" is meant a PPAR subtype protein that is substantially homologous (i.e., no less than 90% homologous in amino acid sequence) to hPPAR α protein, including, but not limited to, hPPAR α .

5 By "NUC protein" is meant a PPAR subtype protein or a PPAR related protein that represses the transcription activation activity of PPAR α protein and/or TR protein, including, but not limited to, hNUC1B protein, hNUC1 protein and proteins with homologous sequences to hNUC1B
10 or hNUC1 protein.

This invention is also directed to compounds, compositions, and methods for modulating processes affected by NUC protein activity and useful for treating a patient exhibiting a pathological condition caused,
15 induced or aggravated by the level of NUC protein activity. More particularly, the invention relates to compounds and pharmaceutical compositions that relieve the repression of PPAR α protein and TR protein activity by a NUC protein.

20 Thus, in one aspect, the present invention features a method for identifying therapeutic agents for treatment of a pathological condition affected by the level of NUC protein activity, comprising the step of screening for an inhibitor of NUC protein activity.

25 One method comprises identifying therapeutic agents which, when added to a system containing NUC protein and PPAR α protein, relieve the repression of PPAR α protein activity by NUC protein.

In a preferred embodiment, this system further
30 contains a reporter gene responsive to PPAR α protein activation, the reduction or relief of the repression of PPAR α by NUC protein is measured by the expression level of the reporter gene.

By "reporter gene" is meant a gene encoding a
35 product that is easily detected and assayed by techniques known to those skilled in the art. A reporter gene in this invention is driven by a promoter that is responsive

to PPAR α protein or TR protein, including, but not limited to, the native promoter of a gene such as acylcoenzyme A oxidase, enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase bifunctional enzyme or 3-ketoacyl thiolase.

5 In a further preferred embodiment, the screening assay is conducted in a cell.

In an even further preferred embodiment, NUC gene, PPAR α gene and the reporter gene are encoded in vectors and introduced into the cell by transfection. The
10 reporter gene has a peroxisome proliferator responsive element (PPRE) and can be activated by PPAR α protein.

In another further preferred embodiment, the screening assay is conducted in an extract of a cell by in vitro transcription.

15 In a third further preferred embodiment, a PPAR activator is added to the screening assay.

By "PPAR activator" is meant a chemical agent that is capable of activating the transcription activation activity of PPAR α protein, such as, but not limited to,
20 CFA (clofibric acid), ETYA (5,8,11,14-eicosatetraynoic acid) or WY-14, 643 ([4-chloro-6-(2,3-xylidino)-2-pyrimidinylthio] acetic acid).

Another method comprises identifying therapeutic agents which, when added to a system containing NUC
25 protein and TR protein, relieve the repression of TR protein activity by NUC protein.

In a preferred embodiment, this system further contains a reporter gene responsive to TR protein activation, the repression or relief of the repression of
30 TR by NUC is measured by the expression level of the reporter gene.

In a further preferred embodiment, the screening assay is conducted in a cell.

In an even further preferred embodiment, NUC gene,
35 TR gene and the reporter gene are encoded in vectors and introduced into the cell by transfection. The reporter gene has a thyroid hormone responsive element (TRE) and

can be activated by TR protein. TREs include, but are not limited to, TREp (palindromic TRE) and DR4 (direct repeat with a 4 nucleotide spacing 5'-AGGTCACAGGAGGTCA-3').

In another further preferred embodiment, the
5 screening assay is conducted in an extract of a cell by in vitro transcription.

In a third further preferred embodiment, a TR activator is added to the screening assay to activate TR.

By "TR activator" is meant a chemical agent that is
10 capable of activating the transcription activation activity of TR protein, such as, but not limited to, LT3 (3,3',5-triiodo-L-thyronine), LT4 (L-thyroxine) or Triac (3,3',5-triiodothyroacetic acid).

A third method comprises identifying agents which,
15 when added to a system containing a NUC protein and a nucleic acid (such as an oligonucleotide) including a PPRE, reduce the binding of NUC protein to the nucleic acid. The level of binding can be detected in a gel retardation assay or other assays known to those skilled
20 in the art.

A fourth method comprises identifying agents which, when added to a system containing a NUC protein and a PPAR α protein, reduce the formation of NUC-PPAR α complexes.

25 In a preferred embodiment, NUC protein is labeled and the formation of NUC-PPAR α complexes is measured by the amount of labeled NUC protein precipitated by PPAR α specific antibody.

A fifth method comprises identifying agents which,
30 when added to a system containing a NUC protein and a TR protein, reduce the formation of NUC-TR complexes.

In a preferred embodiment, NUC protein is labeled and the formation of NUC-TR complexes is measured by the amount of labeled NUC protein precipitated by TR specific
35 antibody.

In another aspect, this invention features a method for treatment of a pathological condition affected by the

level of NUC protein activity by providing an agent that represses or reduces the NUC protein activity. The pathological conditions treated by this method include, but are not limited to, hyperlipidemia, hypercholesteremia
5 and hyperlipoproteinemia.

This invention also relates to novel or unique therapeutic agents discovered by the above methods, i.e., agents that are not known per se or agents that are not already known for use related to treatment of a
10 pathological condition affected by the level of NUC protein activity.

Applicant is particularly interested in the identification of agents of low molecular weight (less than 10,000 daltons, preferably less than 5,000, and most
15 preferably less than 1,000) which can be readily formulated as useful therapeutic agents.

Such agents can then be screened to ensure that they are specific to tissues with pathological conditions induced or aggravated by NUC protein with little or no
20 effect on healthy tissues such that the agents can be used in a therapeutic or prophylactic manner. If such agents have some effect on healthy tissues they may still be useful in therapeutic treatment, particularly in those diseases which are life threatening.

The therapeutic agents discovered by the above assays can then be screened for tissue specificity and toxicity with methods known to those skilled in the art. They can be put in pharmaceutically acceptable
25 formulations, and used for treatment of diseases and pathological conditions induced or aggravated by NUC
30 protein activity.

Once identified, a NUC inhibitor can be used to study the mechanism of NUC inhibition of PPAR α and TR protein activity. Applying techniques known to those
35 skilled in the art, such as those described in J. Sambrook, E. F. Fritsch, and T. Maniatis, Molecular Cloning: A Laboratory Manual, 2 Ed., Cold Spring Harbor

Laboratory Press, Cold Spring Harbor, New York, 1989, a NUC inhibitor can also be used to study the structural changes in NUC protein when the inhibitor binds to the NUC protein, and how the binding affects the ability of the NUC protein to interact with other proteins and to bind to DNA.

Other features and advantages of the invention will be apparent from the following detailed description of the invention, and from the claims.

10 BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 (A-C) is a graph showing normalized luciferase activity. HepG2 cells were transfected with (A) vector pCMVhPPAR α , or pBKCMV (see "Vector construction" in the detailed description of the invention) and treated with CFA. Cells were transfected with pCMVhNUC1B (see "Vector construction" in the detailed description of the invention) or vector and treated with CFA (B) or ETYA (C) and luciferase and β -galactosidase assays performed as described in materials and methods.

20 Figure 2 is a graph showing normalized response of reporter gene to increasing dose of hNUC1B. HepG2 cells were transfected with 0.1 μ g pCMVhPPAR α and increasing amounts of pCMVhNUC1B (indicated in μ gs). CFA was added to a final concentration of 1 mM. Control cells received an equal volume of ethanol (vehicle).

Figure 3 (A-E) is a graph showing normalized response of various receptors to hNUC1B. HepG2 cells were transfected with (A) the ER expression vector HEO (1 μ g) and pLPwtCAT (see "Vector construction" in the detailed description of the invention) (1.5 μ g) reporter (B) pRShRAR α (see "Vector construction" in the detailed description of the invention) and MTV-TREp2-LUC (C) pRShRAR α and CRBP11-tk-LUC (D) pRShTR β (see "Vector construction" in the detailed description of the invention) and MTV-TREp2-LUC. 0.1 μ g of expression plasmid and 0.5 μ g luciferase reporter were used (B-D).

pcMVhNUC1B was co-transfected where indicated, 1 μ g (A) and 0.1 μ g (B-D). The respective ligands for the transfected receptors were β -estradiol (100nM), ATRA (all-trans-retinoic acid) (1 μ M), 9-cis-RA (1 μ M) and L-T3 (100 nM), the final concentration indicated within parentheses. CFA was added where indicated to a final concentration of 1 mM. In Figure 3E, transfections were done as in Figure 3D except that the reporter gene had two copies of DR4 element (direct repeat separated by four nucleotides). The sequence of the DR4 element used is 5'-AGGTCACAGGAGGTCA-3'. The repeat sequence is shown with a line above it.

Figure 4 is a graph showing normalized response of hPPAR α to hNUC1B with increasing ratio of hPPAR α to hNUC1B. HepG2 cells were transfected with 0.05 μ g of pcMVhNUC1B and different amounts of pcMVhPPAR α plasmid (indicated in μ gs). CFA was added to a final concentration of 1 mM.

Figure 5 is a radiograph of a gel retardation assay. DNA binding assays were performed with extracts from COS cells transfected with pcMVhNUC1B or pRShRXR α as described in materials and methods. Extracts from mock transfected cells were used as a control.

DETAILED DESCRIPTION OF THE INVENTION

25 NUC Proteins and Peroxisome Proliferation

The effect of hypolipidemic drugs like gemfibrozil that have significant cardio-protective effect are mediated via the PPARs. To investigate the effect of various fibrates on human PPAR subtypes, we have isolated two human PPAR subtypes, i.e., PPAR α and hNUC1B. Applicant determined that hNUC1B is not activated by PPAR activators such as clofibric acid. Applicant also determined that hNUC1B is a specific repressor of the transcriptional activation effected by PPAR α and thyroid hormone receptor. The repressive action of NUC protein on PPAR α and TR receptors may limit the clinical efficacy of

PPAR α and TR activators (e.g., fibrates, synthroid). Agents that relieve this repression will increase activity of PPAR α and TR and increase the efficacy of existing drugs, or render these drugs unnecessary.

- 5 A subtype of NUC protein, hNUC1, has been shown to be present in the human heart, brain, and liver tissues where PPARs and TRs are active. Therefore, the screening methods of this invention and agents identified thereby may have widespread therapeutic significance.
- 10 We have demonstrated co-operative binding of hNUC1B and RXR α to a PPAR response element, PPRE. Without being bound by any particular theory, applicant proposes that repression of PPAR α by hNUC1B likely occurs at the level of competition for DNA binding, or titrating factors
- 15 required for PPAR α and TR activity. hNUC1B could be binding to the PPRE, thereby antagonizing activation of PPAR α protein. COUP-TF (chicken ovalbumin upstream promoter transcription factor) has been shown to inhibit PPAR activation by a similar mechanism (Miyata, K.S.,
- 20 Zhang, B., Marcus, S.L., Capone, J.P., and Rachubinski, R.A. (1993) Journ. Biol. Chem. 268, 19169-19172). In the absence of a transcription activation function of hNUC1B, this mechanism could explain the repression of PPAR α activity by hNUC1B.
- 25 The invention will now be described in greater detail by reference to the following examples regarding screening for NUC inhibitors. This invention, however, is not limited to co-transfection assay, gel retardation assay and immunoprecipitation assay described below.
- 30 Other methods known to those skilled in the art for assaying an agent that relieve the repressive effect of a protein on a cellular activity may also be used.

Examples

- A candidate agent can be screened by either A)
- 35 indirect evaluation of derepression of a PPAR α or TR responsive gene, B) direct evaluation of NUC protein

binding to a PPAR α or TR responsive element, or C) direct evaluation of complex formation between NUC protein and PPAR α protein or TR protein.

Experimental procedures and reagents employed in the 5 examples described herein are set forth below:

Reagents

ETYA, β -estradiol, ATRA, LT3 (3,3',5-triiodo-L-thyronine) and CFA were purchased from Sigma, and WY-14,643 from Chemsyn Science Laboratories, 10 Lenexa, Kansas, USA. Stock solutions of these compounds were made in ethanol, methanol or dimethyl sulfoxide.

The recipes for buffers, mediums, and solutions in the following examples are given in J. Sambrook, E. F. Fritsch, and T. Maniatis, Molecular Cloning: A Laboratory 15 Manual, 2 Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989.

Vector Construction

For expression in mammalian cells, the hPPAR α cDNA was cloned into the NotI site of pBKCMV (Stratagene) to 20 give pCMVhPPAR α .

The hNUC1B cDNA was directionally cloned into the SalI-SacII site of pBKCMV to give pCMVhNUC1B.

The reporter plasmid pPPRE3-tk-luc was generated by inserting three copies of the synthetic oligonucleotide 25 (5'-CCCGAACGTGACCTTTGTCCTGGTCC-3') containing the "A" site of the Acyl-CoA oxidase gene regulatory sequence (Osumi, T., Wen, J. and Hashimoto, T. (1991) Biochem. Biophys. Res. Commun. 175, 866-871) into the XhoI site 5' of the tk promoter in the previously described pBLtk-luciferase 30 vector (Giguere, V., Hollenberg, S. M., Rosenfeld, M. G., Evans, R. M. (1986) Cell 46, 645-652).

pRShRAR α , pRShRXR α , MTV-TREp2-LUC, and CRBP11-tk-LUC have been described in Giguere, V., Ong, E.S., Segui, P., and Evans, R.M. (1987) Nature 330(2), 624-629; 35 Mangelsdorf, D.J., Ong, E.S., Dyck, J.A., and Evans, R.M.

(1990) Nature 345, 224-229; Umesono, K., Giguere, V., Glass, C.K., and Rosenfeld, M.G. (1988) Nature 336, 262-265 and Mangelsdorf, D.J., Umesono, K., Kleiwer, S.A., Borgmeyer, U., Ong, E.S., and Evans, R.M. (1991) Cell 66, 555-561.

pRShTR β has been described in Thomson, C.C., and Evans, R.M. (1989) Proc. Natl. Acad. Sci. USA. 86, 3494-3498.

The human TR α 1 cDNA (Nakai, A., Sakurai, A., Bell, G.I., and DeGroot, L.J. (1988) Molec. Endoc. 2, 1087-1092) was liberated from pME21 by digestion with EcoR1 and blunt ended by digestion with mung bean nuclease. pRS plasmid (Giguere, V., Hollenberg, S. M., Rosenfeld, M. G., Evans, R. M. (1986) Cell 46, 645-652) was digested with BamH1, dephosphorylated and repaired with Klenow enzyme. The TR α 1 cDNA was then joined to the vector by blunt end ligation.

The ER expression plasmid HEO has been described in (Kumar, V. and Chambon, P. (1988) Cell 55, 145-156).

The estrogen inducible brain creatine kinase promoter was cloned into pUCPLCAT to give pLPwtCAT.

Co-transfection Assay

HepG2 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10%(v/v) fetal bovine serum (Hyclone), 2 mM L-glutamine, and 55 μ g/ml gentamicin (BioWhittaker). Cells were plated at 2×10^5 cells per well for HepG2 in 12 well cell culture dishes (Costar). The media was replaced with fresh media 20 hours later. After 4 hours, DNA was added by the calcium phosphate coprecipitation technique (Berger, T. S., Parandosh, Z., Perry, B., and Stein, R.B. (1992) J. Steroid. Biochem. Molec. Biol. 41, 733-738). Typically, 0.1 μ g of expression plasmid, 0.5 μ g of the β -gal expression plasmid pCH110 (internal control), and 0.5 mg of reporter plasmid were added to each well.

Where indicated, 0-0.5 μ g of hNUC1B plasmid

(repressor) was added. Repressor plasmid dosage was kept constant by the addition of appropriate amounts of the empty expression vector pBKCMV. Total amount of DNA was kept at 20 μ g by the addition of pGEM DNA (Promega).

5 After 14 hours the cells were washed with 1X PBS and fresh media added (DMEM with 10% charcoal stripped fetal bovine serum (Hyclone) plus the above supplements). Ligands or PPAR activators were added to the final concentrations indicated. Control cells were treated with
10 vehicle.

 After another 24 hours the cells were harvested and the luciferase and β -galactosidase activities quantified on a Dynatech ML 1000 luminometer and a Beckman Biomek 1000 workstation respectively. The normalized response is
15 the luciferase activity of the extract divided by the β -galactosidase activity of the same. Each data point represents the mean of three transfections. Error bars represent the standard deviation from the mean. CAT assays were performed as in Ausbel, F.M., Brent, R.,
20 Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K. (1987) in Current Protocols in Molecular Biology, Wiley Interscience.

Gel Retardation Assay

 COS cells were transfected with 5 μ g of pCMVhNUC1B
25 or pRShRXR α (Ptashne, M. (1988) Nature 335, 683-689) per 100 mm dish for 48 hours. Whole cell extracts were made by four cycles of freeze-thawing in 0.4 M KCl containing buffer followed by centrifugation. Gel retardations were performed by incubating 5 μ g of cell extract in buffer
30 containing 10 mM Hepes (7.8), 50 mM KCl, 1 mM DTT, 2.5 mM MgCl₂, 0.5mg/ml dIdC and 20% glycerol at 4°C for 5 minutes. About 100,000 cpm of ³²P-end-labeled probe was then added and incubated at 25°C for another 5 minutes.

 Protein-DNA complexes were resolved by
35 electrophoresis on 5% polyacrylamide gels in 0.5X TBE. The PPRE sequence from the acyl-coenzymeA oxidase (AOX)

gene used as probe is

5'-CTAGCGATATCATGACCTTTGTCCTAGGCCTC-3' (upper strand) and

5'-CTAGGAGGCCTAGGACAAAGGTCATGATATCG-3' (lower strand).

Example 1. Cloning of hPPAR α

5 A human homologue of rat PPAR α was isolated from a
human liver 5'-stretch lgt10 cDNA library (Clontech). The
library was screened at medium stringency (40% formamide,
5X SSC at 37°C), with a rPPAR nick translated DNA fragment
specific to the A/B and DNA binding domain (from the EcoR1
10 to the BglII site, nucleotides 450-909) (Gottlicher, M.,
Widmar, E., Li, Q., and Gustafsson, J. A. (1992) Proc.
Natl. Acad. Sci. USA. 89, 4653-4657). Positive clones
were isolated and subcloned into the Bluescript KS vector
(Stratagene) for sequencing. The sequence is identical to
15 that published by Sher et. al., (Sher, T., Yi, H. F.,
McBride, W. O. and Gonzales., F. J. (1993) Biochemistry
32, 5598-5604) except for two amino acid differences,
alanine at position 268 and glycine at position 296.

20 The activation profile of hPPAR α by CFA is shown in
Fig. 1A. This receptor is also activated by other known
activators of PPARs, e.g., WY-14,643 and ETYA in HepG2 and
CV-1 cells.

Example 2. Cloning of hNUC1B

25 hNUC1B was isolated from a human kidney cDNA library
by screening with a probe specific to the rat PPAR DNA
binding domain (from the PvuII to the BglII site,
nucleotides 618-909, reference (Gottlicher, M., Widmar,
E., Li, Q., and Gustafsson, J. A. (1992) Proc. Natl. Acad.
Sci. USA. 89, 4653-4657)) using procedures as described
30 above in Example 1. A recombinant clone was isolated,
subcloned into pGEM-5Zf and sequenced. The sequence of
this receptor is identical to that of the hNUC1 sequence
(Schmidt, A., N. Endo, S. J. Rutledge, R. Vogel, D.
Shinar, and G. A. Rodan. (1992) Mol. Endocrinol. 6,
35 1634-1641) except for alanine at position 292.

hNUC1B is a member of the PPAR family. hNUC1B has 61% homology to hPPAR α and the two cysteine residues in the "D" box are separated by three amino acids (E, R and S, positions 112-114 of the amino acid sequence). This is a characteristic of PPARs (Dreyer, C., Krey, G., Hansjorg, K., Givel, F., Helftenbein, G., and Wahli, W. (1992) Cell 68, 879-887). All the other nuclear receptors have five amino acids in the same region.

The hNUC1B protein, unlike hPPAR α , is not transcriptionally activated in HepG2 or CV-1 cells by CFA, ETYA or WY-14,643 (Fig. 1B, C). The slight activation seen in the absence of transfected receptor is probably due to the endogenous PPARs in the cell line utilized. This has also been observed by Schmidt, A., N. Endo, S. J. Rutledge, R. Vogel, D. Shinar, and G. A. Rodan. (1992) Mol. Endocrinol. 6, 1634-1641 with hNUC1 and certain fatty acids. This data suggests the absence of a PPAR activator inducible transactivation function in hNUC1B.

Transfected hNUC1B, however, did decrease the response from the endogenous PPARs. This suggested that hNUC1B may act as a repressor of hPPAR function. Figure 4 shows that increasing ratio of hPPAR α to hNUC1B overcame the repression by hNUC1B.

Example 3. Screening for hNUC1B Inhibitors with Co-transfection Assay

Increasing amounts of hNUC1B plasmid were co-transfected with a constant amount of hPPAR α expressing plasmid into cells. Figure 2 shows a strong dose dependent repression of hPPAR α activity by hNUC1B in the presence of CFA. Repression was 85% with 0.1 μ g of cotransfected hNUC1B plasmid. Repression by hNUC1 was also observed on the rat PPAR (Gottlicher, M., Widmar, E., Li, Q., and Gustafsson, J. A. (1992) Proc. Natl. Acad. Sci. USA. 89, 4653-4657) and on hPPAR α in the presence of ETYA and WY-14,643.

To determine whether hNUC1B is a specific repressor

of hPPAR α , we tested the effect of hNUC1B on other members of the steroid receptor family (Fig. 3 A-C).

hNUC1B has minimal effect on activation of ER and RAR α by their respective ligands. hNUC1B does not repress
5 RXR α in the absence of CFA and only 25% repression was detected in its presence.

However, with hTR β 1 and a palindromic TRE, 65% repression by hNUC1 was observed in the absence of CFA (Fig. 3 D). Repression increased to 75% in the presence
10 of CFA. Repression was also observed with hTR α , although to a lesser degree.

Therefore, hNUC1B is not a general transcription repressor, but a dominant negative repressor of hPPAR α and hTR. Repression occurred in the absence of clofibric
15 acid, but was enhanced in its presence.

In order to screen for agents that relieve the repression PPAR α and TR activity by hNUC1B, PPAR α and hNUC1B or TR and hNUC1B expressing plasmids will be
20 HepG2 (a human liver cell line) cells along with a reporter containing PPAR or TR binding elements (such as PPRES, or TREs) in the presence of a PPAR activator (e.g., clofibric acid, WY-14,643) or a TR activator (e.g., LT3).

Clofibric acid or LT3 normally activate their
25 respective receptors and will therefore give a strong signal. In the presence of hNUC1B the signal will be very weak because of repression of these receptors by hNUC1B. We will add compounds to the transfected cells at various concentrations and select those that relieve the
30 repression by hNUC1B.

The above screening strategy will also be followed in a yeast based assay with appropriate vectors and reporters.

Example 4. Screening for hNUC1B Inhibitors by Gel 35 Retardation Assay

Gel retardation assays showed that hNUC1B binds to

a PPAR element, PPRE. With hNUC1B or hRXR α alone, weak retarded complexes were seen (Fig. 5, lanes 1 and 2). Addition of RXR α enhances binding of hNUC1B (lane 3), demonstrating co-operative binding of hNUC1B and hRXR α to the PPRE. Similar results have been observed with mPPAR α and rPPAR (Kliewer, S. A., Umesono, K., Noonan, D. J., Heyman, R. A., and Evans, R. M. (1992) Nature (London) 358, 771-774; and Isseman, I., Prince, R.A., Tugwood, J.D., and Green, S. (1993) Biochemie 75, 251-256. Gearing, K. L., Gottlicher, M., Teboul, M., Widmark, E., and Gustafsson., (1993) J. Proc. Natl. Acad. Sci. USA. 90, 1440-1444).

Since hNUC1B could be repressing the activation of PPAR α or TR simply by binding to the DNA sequences normally bound by these receptors, we will screen for compounds that prevent formation of a hNUC1B-PPRE complex by adding them during the binding reaction. Similar reaction assays will be done with TR α and TR β .

Example 5. Screening for hNUC1B Inhibitors with Immuno-precipitation assay

hNUC1B may simply dimerize with PPAR α or TR to form an inactive heterodimer. To screen for agents that relieve the repression PPAR α and TR activity by hNUC1B, we will mix labeled hNUC1B with unlabeled TR or PPAR α . TR or PPAR α specific antibodies will then be used to immunoprecipitate the hNUC1B-TR or hNUC1B-PPAR α complexes respectively. Test compounds will be added to this mix and only those that disrupt the formation of these heterodimers will be selected. These compounds will then be further tested by the methods described above to see if they relieve repression of PPAR and TR by hNUC1B.

Example 6. Toxicity-testing of Putative NUC Inhibitors

Methods are provided for determining whether an agent active in any of the methods listed above has little or no effect on healthy cells. Such agents are then

formulated in a pharmaceutically acceptable buffer or in buffers useful for standard animal tests.

By "pharmaceutically acceptable buffer" is meant any buffer which can be used in a pharmaceutical composition prepared for storage and subsequent administration, which comprise a pharmaceutically effective amount of an agent as described herein in a pharmaceutically acceptable carrier or diluent. Acceptable carriers or diluents for therapeutic use are well known in the pharmaceutical art, and are described, for example, in Remington's Pharmaceutical Sciences, Mack Publishing Co. (A.R. Gennaro edit. 1985). Preservatives, stabilizers, dyes and even flavoring agents may be provided in the pharmaceutical composition. For example, sodium benzoate, sorbic acid and esters of p-hydroxybenzoic acid may be added as preservatives. Id. at 1449. In addition, antioxidants and suspending agents may be used. Id.

A. Additional screens for Toxicity: Method 1

Putative NUC inhibitors are assessed for toxicity to cultured human cells. This assessment is based on the ability of living cells to reduce 2,3,-bis[2-methoxy-4-nitro-5-sulphonylphenyl]-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide) otherwise referred to as XTT (Paull et al., J. Heterocycl. Chem. 25:763-767 (1987); Weislow et al., (1989), J. Natl. Canc. Inst. 81:577).

Viable mammalian cells are capable of reductive cleavage of an N-N bond in the tetrazole ring of XTT to form XTT formazan. Dead cells or cells with impaired energy metabolism are incapable of this cleavage reaction. The extent of the cleavage is directly proportional to the number of living cells tested.

Cells from a human cell line such as HeLa cells are seeded at 10^3 per well in 0.1 ml of cell culture medium (Dulbecco's modified minimal essential medium supplemented with 10% fetal calf serum) in the wells of a 96 well microtiter plate. Cells are allowed to adhere to the plate by culture at 37° C in an atmosphere of 95% air, 5%

CO₂.

After overnight culture, solutions of test substances are added in duplicate to wells at concentrations that represent eight half-decade log dilutions. In parallel, the solvent used to dissolve the test substance is added in duplicate to other wells. The culture of the cells is continued for a period of time, typically 24 hours.

At the end of that time, a solution of XTT and a coupler (methylphenazonium sulfate) is added to each of the test wells and the incubation is continued for an additional 4 hours before the optical density in each of the wells is determined at 450 nm in an automated plate reader. Substances that kill mammalian cells, or impair their energy metabolism, or slow their growth are detected by a reduction in the optical density at 450 nm in a well as compared to a well which received no test substance.

B. Additional screens for Toxicity: Method 2

Putative NUC inhibitors are tested for cytotoxic effects on cultured human cell lines using incorporation of ³⁵S methionine into protein as an indicator of cell viability.

HeLa cells are grown in 96 well plates in Dulbecco's minimal essential medium supplemented with 10% fetal calf serum and 50 µg/ml penicillin and streptomycin. Cells are initially seeded at 10³ cells/well, 0.1 ml/well. Cells are grown for 48 hrs without exposure to the NUC inhibitor, then medium is removed and varying dilutions of the NUC inhibitor prepared in complete medium are added to each well, with control wells receiving no NUC inhibitor.

Cells are incubated for an additional 48-72 hrs. Medium is changed every 24 hrs and replaced with fresh medium containing the same concentration of the NUC inhibitors. Medium is then removed and replaced with complete medium without NUC inhibitor.

Cells are incubated for 24 hr in the absence of NUC inhibitor, then viability is estimated by the incor-

poration of ^{35}S into protein. Medium is removed, replaced with complete medium without methionine, and incubated for 30 min. Medium is again removed, and replaced with complete medium without methionine but containing 0.1 $\mu\text{Ci/ml}$ ^{35}S methionine. Cells are incubated for 3 hrs.

Wells are washed 3 times in PBS, then cells are permeabilized by adding 100% methanol for 10 min. Ice cold 10% trichloroacetic acid (TCA) is added to fill wells; plates are incubated on ice for 5 min. This TCA wash is repeated two more times. Wells are again washed in methanol, then air dried. 50 μl of scintillation cocktail are added to each well and dried onto the wells by centrifugation. Plates are used to expose X ray film. Densitometer scanning of the autoradiogram, including wells without NUC inhibitor, is used to determine the dosage at which 50% of cells are not viable (ID_{50}) (Culture of Animal Cells. A manual of basic technique. (1987). R. Ian Freshney. John Wiley & Sons, Inc., New York).

Example 7. Pharmaceutical Formulations and Administration of NUC Inhibitor

The particular agent that affects hNUC1 activity and the pathological condition of interest can be administered to a patient either by themselves, or in pharmaceutical compositions where it is mixed with suitable carriers or excipient(s).

In treating a patient exhibiting a pathological condition induced or aggravated by the level of hNUC1 activity, a therapeutically effective amount of a agent or agents such as these is administered. By "therapeutically effective amount" is meant an amount that relieves (to some extent) one or more symptoms of the disease or condition in the patient. Additionally, by "therapeutically effective amount" is meant an amount that returns to normal, either partially or completely, physiological or biochemical parameters associated with or causative of a disease or condition.

Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., for determining the LD_{50} (the dose lethal to 50% of the population) and the ED_{50} (the dose therapeutically effective in 50% of the population). Each candidate compound is tested for its efficacy in relieving the repression of PPAR α and TR by hNUC1 in cell lines, in animal models, and in controlled clinical studies using methods known to those skilled in the art and approved by the Food and Drug Administration, such as, but not limited to, those promulgated in the Federal Register 47 (no. 56) : 12558-12564, March 23, 1982. The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD_{50}/ED_{50} . Compounds which exhibit large therapeutic indices are preferred. The data obtained from these cell culture assays and animal studies can be used in formulating a range of dosage for use in human. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED_{50} with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized.

For any compound used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. For example, a dose can be formulated in animal models to achieve a circulating plasma concentration range that includes the IC_{50} as determined in cell culture (i.e., the concentration of the test compound which achieves a half-maximal disruption of the protein complex, or a half-maximal inhibition of the cellular level and/or activity of a complex component). Such information can be used to more accurately determine useful doses in humans. Levels in plasma may be measured, for example, by HPLC.

The exact formulation, route of administration and dosage can be chosen by the individual physician in view

of the patient's condition. (See e.g. Fingl et al., in The Pharmacological Basis of Therapeutics, 1975, Ch. 1 p. 1). It should be noted that the attending physician would know how to and when to terminate, interrupt, or
5 adjust administration due to toxicity, or to organ dysfunctions. Conversely, the attending physician would also know to adjust treatment to higher levels if the clinical response were not adequate (precluding toxicity). The magnitude of an administered dose in the management
10 of the cardiovascular disorder of interest will vary with the severity of the condition to be treated and to the route of administration. The severity of the condition may, for example, be evaluated, in part, by standard prognostic evaluation methods. Further, the dose and
15 perhaps dose frequency, will also vary according to the age, body weight, and response of the individual patient. A program comparable to that discussed above may be used in veterinary medicine.

Depending on the specific conditions being treated,
20 such agents may be formulated and administered systemically or locally. Techniques for formulation and administration may be found in Remington's Pharmaceutical Sciences, 18th ed., Mack Publishing Co., Easton, PA (1990). Suitable routes may include oral, rectal,
25 transdermal, vaginal, transmucosal, or intestinal administration; parenteral delivery, including intramuscular, subcutaneous, intramedullary injections, as well as intrathecal, direct intraventricular, intravenous, intraperitoneal, intranasal, or intraocular injections,
30 just to name a few.

For injection, the agents of the invention may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks's solution, Ringer's solution, or physiological saline buffer. For
35 such transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

Use of pharmaceutically acceptable carriers to formulate the compounds herein disclosed for the practice of the invention into dosages suitable for systemic administration is within the scope of the invention. With proper choice of carrier and suitable manufacturing practice, the compositions of the present invention, in particular, those formulated as solutions, may be administered parenterally, such as by intravenous injection. The compounds can be formulated readily using pharmaceutically acceptable carriers well known in the art into dosages suitable for oral administration. Such carriers enable the compounds of the invention to be formulated as tablets, pills, capsules, liquids, gels, syrups, slurries, suspensions and the like, for oral ingestion by a patient to be treated.

Agents intended to be administered intracellularly may be administered using techniques well known to those of ordinary skill in the art. For example, such agents may be encapsulated into liposomes, then administered as described above. Liposomes are spherical lipid bilayers with aqueous interiors. All molecules present in an aqueous solution at the time of liposome formation are incorporated into the aqueous interior. The liposomal contents are both protected from the external microenvironment and, because liposomes fuse with cell membranes, are efficiently delivered into the cell cytoplasm. Additionally, due to their hydrophobicity, small organic molecules may be directly administered intracellularly.

Pharmaceutical compositions suitable for use in the present invention include compositions wherein the active ingredients are contained in an effective amount to achieve its intended purpose. Determination of the effective amounts is well within the capability of those skilled in the art, especially in light of the detailed disclosure provided herein.

In addition to the active ingredients, these pharmaceutical compositions may contain suitable pharma-

ceutically acceptable carriers comprising excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically. The preparations formulated for oral administration may be in the form of tablets, dragees, capsules, or solutions.

The pharmaceutical compositions of the present invention may be manufactured in a manner that is itself known, e.g., by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping or lyophilizing processes.

Pharmaceutical formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form. Additionally, suspensions of the active compounds may be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Optionally, the suspension may also contain suitable stabilizers or agents which increase the solubility of the compounds to allow for the preparation of highly concentrated solutions.

Pharmaceutical preparations for oral use can be obtained by combining the active compounds with solid excipient, optionally grinding a resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries, if desired, to obtain tablets or dragee cores. Suitable excipients are, in particular, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carboxymethylcellulose, and/or polyvinylpyrrolidone (PVP). If

desired, disintegrating agents may be added, such as the cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate.

Dragee cores are provided with suitable coatings.

- 5 For this purpose, concentrated sugar solutions may be used, which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or
10 pigments may be added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses.

- Pharmaceutical preparations which can be used orally include push-fit capsules made of gelatin, as well as
15 soft, sealed capsules made of gelatin and a plasticizer, such as glycerol or sorbitol. The push-fit capsules can contain the active ingredients in admixture with filler such as lactose, binders such as starches, and/or lubricants such as talc or magnesium stearate and, option-
20 ally, stabilizers. In soft capsules, the active compounds may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilizers may be added.

- All publications referenced are hereby incorporated
25 by reference herein, including the nucleic acid sequences and amino acid sequences listed in each publication.

Other embodiments are within the following claims.

26

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Mukherjee, Ranjan
- (ii) TITLE OF INVENTION: SCREENING FOR NUC INHIBITOR
- (iii) NUMBER OF SEQUENCES: 6
- (iv) CORRESPONDENCE ADDRESS:
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Suite 4700
- (C) CITY: Los Angeles
- (D) STATE: California
- (E) COUNTRY: U.S.A.
- (F) ZIP: 90071-2066
- (v) COMPUTER READABLE FORM:
- (A) MEDIUM TYPE: 3.5" Diskette
1.44 Mb storage
- (B) COMPUTER: IBM Compatible
- (C) OPERATING SYSTEM: IBM P.C. DOS 5.0
- (D) SOFTWARE: Word Perfect 5.1
- (vi) CURRENT APPLICATION DATA:
- (A) APPLICATION NUMBER: 08/270,635
- (B) FILING DATE: July 1, 1994
- (C) CLASSIFICATION: 514
- (vii) PRIOR APPLICATION DATA:
- Prior applications total,
including application

27

described below: 2

(A) APPLICATION NUMBER: 08/141,500
(B) FILING DATE: October 22, 1993
(A) APPLICATION NUMBER: 08/143,215
(B) FILING DATE: October 25, 1993

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Warburg, Richard J.
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(C) REFERENCE/DOCKET NUMBER: 207/200

(ix) TELECOMMUNICATION INFORMATION:

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(B) TELEFAX: (213) 955-0440
(C) TELEX: 67-3510

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1326 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

ATGGAGCAGC CACAGGAGGA AGCCCCTGAG GTCCGGGAAG AGGAGGAGAA AGAGGAAGTG 60
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AGCTACACAG ACCTCTCCCG GAGCTCCTCG CCACCCTCAC TGCTGGACCA ACTGCAGATG 180
GGCTGTGACG GGGCCTCATG CGGCAGCCTC AACATGGAGT GCCGGGTGTG CGGGGAACAAG 240

GCATCGGGCT TCCACTACGG TGTTTCATGCA TGTGAGGGGT GCAAGGGCTT CTTCCGTCGT 300
ACGATCCGCA TGAAGCTGGA GTACGAGAAG TGTGAGCGCA GCTGCAAGAT TCAGAAGAAG 360
AACCGCAACA AGTGCCAGTA CTGCCGCTTC CAGAAGTGCC TGGCACTGGG CATGTCACAC 420
AACGCTATCC GTTTTGGTCG GATGCCGGAG GCTGAGAAGA GGAAGCTGGT GGCAGGGCTG 480
ACTGCAAACG AGGGGAGCCA GTACAACCCA CAGGTGGCCG ACCTGAAGGC CTTCTCCAAG 540
CACATCTACA ATGCCTACCT GAAAACTTC AACATGACCA AAAAGAAGGC CCGCAGCATC 600
CTCACCGGCA AAGCCAGCCA CACGGCGCCC TTTGTGATCC ACGACATCGA GACATTGTGG 660
CAGGCAGAGA AGGGGCTGGT GTGGAAGCAG TTGGTGAATG GCCTGCCTCC CTACAAGGAG 720
ATCAGCGTGC ACGTCTTCTA CCGCTGCCAG TGCACCACAG TGGAGACCGT GCGGGAGCTC 780
ACTGAGTTCTG CCAAGAGCAT CCCAGCTTC AGCAGCCTCT TCCTCAACGA CCAGGTTACC 840
CTTCTCAAGT ATGGCGTGCA CGAGGCCATC TTCGCCATGC TGGCCTCTAT CGTCAACAAG 900
GACGGGCTGC TGGTAGCCAA CGGCAGTGGC TTTGTCACCC GTGAGTTCCT GCGCAGCCTC 960
CGCAAACCCT TCAGTGATAT CATTGAGCCT AAGTTTGAAT TTGCTGTCAA GTTCAACGCC 1020
CTGGAATTG ATGACAGTGA CCTGGCCCTA TTCATTGCGG CCATCATTCT GTGTGGAGAC 1080
CGGCCAGGCC TCATGAACGT TCCACGGGTG GAGGCTATCC AGGACACCAT CCTGCGTGCC 1140
CTCGAATTCC ACCTGCAGGC CAACCACCCT GATGCCCAGT ACCTCTTCCC CAAGCTGCTG 1200
CAGAAGATGG CTGACCTGCG GCAACTGGTC ACCGAGCACG CCCAGATGAT GCAGCGGATC 1260
AAGAAGACCG AAACCGAGAC CTCGCTGCAC CCTCTGCTCC AGGAGATCTA CAAGGACATG 1320
TACTAA 1326

29

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 441 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION : SEQ ID NO: 2

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Met Glu Gln Pro Gln Glu Glu Ala Pro Glu Val Arg Glu Glu Glu Glu
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Lys Glu Glu Val Ala Glu Ala Glu Gly Ala Pro Glu Leu Asn Gly Gly
      20             25             30

Pro Gln His Ala Leu Pro Ser Ser Ser Tyr Thr Asp Leu Ser Arg Ser
      35             40             45

Ser Ser Pro Pro Ser Leu Leu Asp Gln Leu Gln Met Gly Cys Asp Gly
      50             55             60

Ala Ser Cys Gly Ser Leu Asn Met Glu Cys Arg Val Cys Gly Asp Lys
65             70             75             80

Ala Ser Gly Phe His Tyr Gly Val His Ala Cys Glu Gly Cys Lys Gly
      85             90             95

Phe Phe Arg Arg Thr Ile Arg Met Lys Leu Glu Tyr Glu Lys Cys Glu
      100            105            110

Arg Ser Cys Lys Ile Gln Lys Lys Asn Arg Asn Lys Cys Gln Tyr Cys
      115            120            125

Arg Phe Gln Lys Cys Leu Ala Leu Gly Met Ser His Asn Ala Ile Arg

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30

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145	150	155 160
Thr Ala Asn Glu Gly Ser Gln Tyr Asn Pro Gln Val Ala Asp Leu Lys		
	165	170 175
Ala Phe Ser Lys His Ile Tyr Asn Ala Tyr Leu Lys Asn Phe Asn Met		
	180	185 190
Thr Lys Lys Lys Ala Arg Ser Ile Leu Thr Gly Lys Ala Ser His Thr		
	195	200 205
Ala Pro Phe Val Ile His Asp Ile Glu Thr Leu Trp Gln Ala Glu Lys		
	210	215 220
Gly Leu Val Trp Lys Gln Leu Val Asn Gly Leu Pro Pro Tyr Lys Glu		
225	230	235 240
Ile Ser Val His Val Phe Tyr Arg Cys Gln Cys Thr Thr Val Glu Thr		
	245	250 255
Val Arg Glu Leu Thr Glu Phe Ala Lys Ser Ile Pro Ser Phe Ser Ser		
	260	265 270
Leu Phe Leu Asn Asp Gln Val Thr Leu Leu Lys Tyr Gly Val His Glu		
	275	280 285
Ala Ile Phe Ala Met Leu Ala Ser Ile Val Asn Lys Asp Gly Leu Leu		
	290	295 300
Val Ala Asn Gly Ser Gly Phe Val Thr Arg Glu Phe Leu Arg Ser Leu		
305	310	315 320
Arg Lys Pro Phe Ser Asp Ile Ile Glu Pro Lys Phe Glu Phe Ala Val		
	325	330 335

31

Lys Phe Asn Ala Leu Glu Leu Asp Asp Ser Asp Leu Ala Leu Phe Ile
 340 345 350

Ala Ala Ile Ile Leu Cys Gly Asp Arg Pro Gly Leu Met Asn Val Pro
 355 360 365

Arg Val Glu Ala Ile Gln Asp Thr Ile Leu Arg Ala Leu Glu Phe His
 370 375 380

Leu Gln Ala Asn His Pro Asp Ala Gln Tyr Leu Phe Pro Lys Leu Leu
 385 390 395 400

Gln Lys Met Ala Asp Leu Arg Gln Leu Val Thr Glu His Ala Gln Met
 405 410 415

Met Gln Arg Ile Lys Lys Thr Glu Thr Glu Thr Ser Leu His Pro Leu
 420 425 430

Leu Gln Glu Ile Tyr Lys Asp Met Tyr
 435 440

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 16 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

AGGTCACAGG AGGTCA

16

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

32

(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

CCCGAACGTG ACCTTTGTCC TGGTCC

26

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

CTAGCGATAT CATGACCTTT GTCCTAGGCC TC

32

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

CTAGGAGGCC TAGGACAAAG GTCATGATAT CG

32

WHAT IS CLAIMED IS:

1. Method for screening for a therapeutic agent for treatment of a pathological condition affected by the level of NUC protein activity, comprising the step of
5 screening for an inhibitor of NUC protein activity.
2. The method of claim 1, wherein said screening comprising the steps of:
contacting a potential agent with a system comprising a NUC protein and a PPAR α protein, wherein the
10 activity of said PPAR α protein is repressed or reduced by said NUC protein;
measuring the activity of said PPAR α protein; wherein an increase in the activity of said PPAR α protein compared to the activity in the absence of said agent is
15 an indication that said agent is potentially useful for treatment of said condition.
3. The method of claim 2, wherein said system further comprises a reporter gene whose expression is activated by said PPAR α protein activity; and said PPAR α
20 protein activity is measured by the expression level of said reporter gene.
4. The method of claim 3, wherein said system comprises a cell.
5. The method of claim 4, wherein said NUC protein
25 is expressed from a vector transfected into said cell.
6. The method of claim 4, wherein said PPAR α protein is expressed from a vector transfected into said cell.
7. The method of claim 4, wherein said reporter
30 gene is transfected into said cell in a vector.

8. The method of claim 3, wherein said system comprises an extract of a cell.

9. The method of claim 3, wherein said system further comprises a PPAR activator.

5 10. The method of claim 9, wherein said activator is selected from a group consisting of CFA, ETYA, and WY-14, 643.

11. The method of claim 3, wherein said reporter gene comprises a PPRE element.

10 12. The method of claim 2, wherein said NUC protein consists of a hNUC1B protein.

13. The method of claim 2, wherein said NUC protein consists of a hNUC1 protein.

15 14. The method of claim 1, wherein said screening comprising the steps of:

contacting a potential agent with a system comprising a NUC protein and a TR protein, wherein the activity of said TR protein is repressed or reduced by said NUC protein;

20 measuring the activity of said TR protein; wherein an increase in the activity of said TR protein compared to the activity in the absence of said agent is an indication that said agent is potentially useful for treatment of said condition.

25 15. The method of claim 14, wherein said system further comprises a reporter gene whose expression is activated by said TR protein activity; and said TR protein activity is measured by the expression level of said reporter gene.

16. The method of claim 15, wherein said system comprises a cell.

17. The method of claim 16, wherein said NUC protein is expressed from a vector transfected into said
5 cell.

18. The method of claim 16, wherein said TR protein is expressed from a vector transfected into said cell.

19. The method of claim 16, wherein said reporter gene is transfected into said cell in a vector.

10 20. The method of claim 15, wherein said system comprises an extract of a cell.

21. The method of claim 15, wherein said system further comprises a TR activator.

22. The method of claim 21, wherein said activator
15 is selected from a group consisting of LT3, LT4 and Triac.

23. The method of claim 15, wherein said reporter gene comprises a TRE element.

24. The method of claim 14, wherein said NUC protein consists of a hNUC1B protein.

20 25. The method of claim 14, wherein said NUC protein consists of a hNUC1 protein.

26. The method of claim 1, wherein said screening comprising the steps of:

25 contacting a potential agent with a system comprising a NUC protein and a nucleic acid comprising a PPRE element, wherein said NUC protein binds to said nucleic acid to form a complex;

measuring the level of binding between said NUC protein and said nucleic acid; wherein a reduction in the binding compared to the binding in the absence of said agent is an indication that said agent is potentially
5 useful for treatment of said condition.

27. The method of claim 26, wherein said measuring comprises determining the ratio of said nucleic acid in said complex and said nucleic without said complex.

28. The method of claim 26, wherein said NUC
10 protein consists of a hNUC1B protein.

29. The method of claim 26, wherein said NUC protein consists of a hNUC1 protein.

30. The method of claim 1, wherein said screening comprising the steps of:
15 contacting a potential agent with a system comprising a NUC protein and a PPAR α protein, wherein said NUC protein binds to said PPAR α protein to form a NUC-PPAR α complex;
measuring the level of said NUC-PPAR α complex;
20 wherein a reduction in the level of said NUC-PPAR α complex compared to the level in the absence of said agent is an indication that said agent is potentially useful for treatment of said condition.

31. The method of claim 30, wherein said system
25 further comprises a PPAR α antibody, and said measuring comprises determining the level of said NUC-PPAR α complex precipitated by said PPAR α antibody.

32. The method of claim 31, wherein said NUC protein is labeled with a radioactive element.

30 33. The method of claim 30, wherein said NUC

37

protein consists of a hNUC1B protein.

34. The method of claim 30, wherein said NUC protein consists of a hNUC1 protein.

35. The method of claim 1, wherein said screening
5 comprising the steps of:

contacting a potential agent with a system comprising a NUC protein and a TR protein, wherein said NUC protein binds to said TR protein to form a NUC-PPAR α complex;

10 measuring the level of said NUC-TR complex; wherein a reduction in the level of said NUC-TR complex compared to the level in the absence of said agent is an indication that said agent is potentially useful for treatment of said condition.

15 36. The method of claim 35, wherein said system further comprises a TR antibody, and said measuring comprises determining the level of said NUC-TR complex precipitated by said TR antibody.

20 37. The method of claim 36, wherein said NUC protein is labeled with a radioactive element.

38. The method of claim 35, wherein said NUC protein consists of a hNUC1B protein.

39. The method of claim 35, wherein said NUC protein consists of a hNUC1 protein.

25 40. Method for treatment of a pathological condition affected by the level of NUC protein activity, comprising the step of providing an agent that represses or reduces said NUC protein activity.

41. The method of claim 40, wherein said

pathological condition is selected from a group consisting of hyperlipidemia, hypercholesteremia and hyperlipoproteinemia.

42. Purified nucleic acid comprising the nucleotide
5 sequence shown in SEQ ID NO. 1.

43. A vector comprising said nucleic acid of claim
42.

44. Recombinant hNUC1B protein expressed from said
nucleic acid of claim 42.

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Figure 1

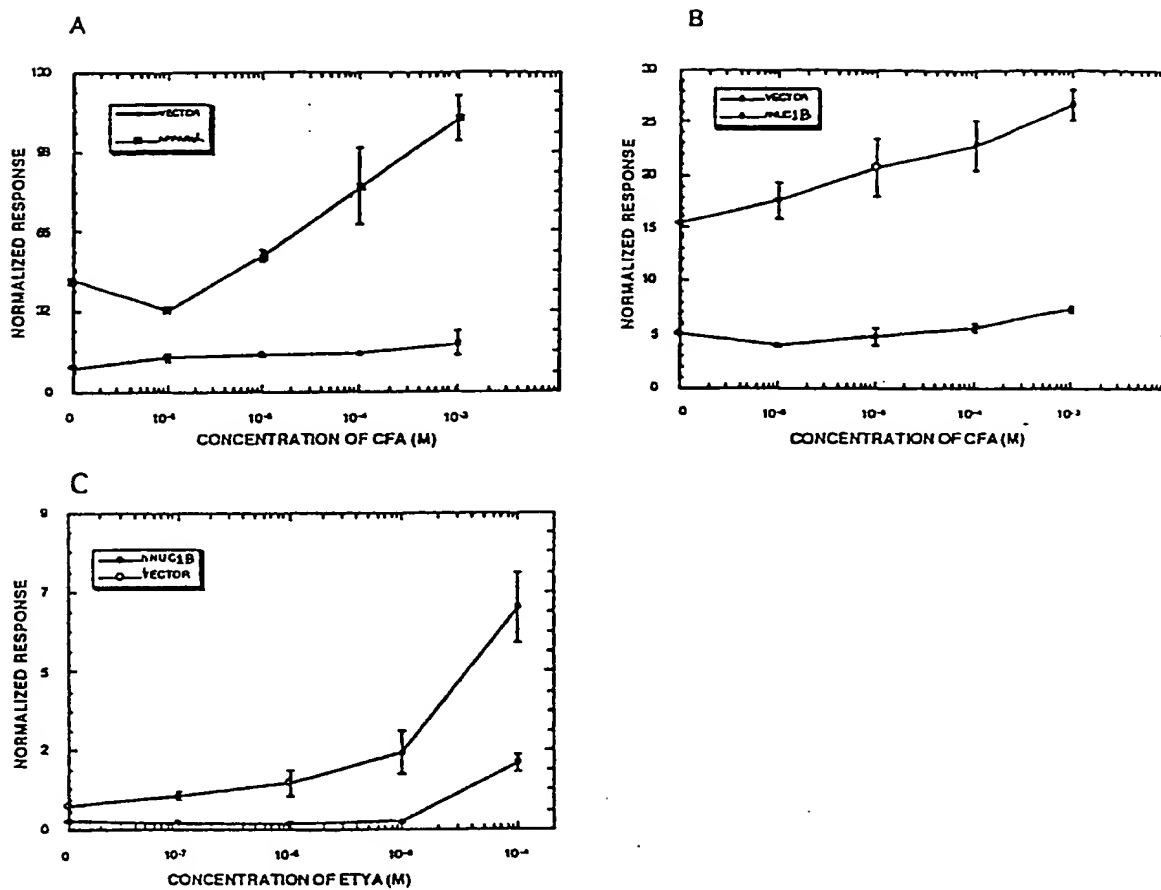


Figure 2

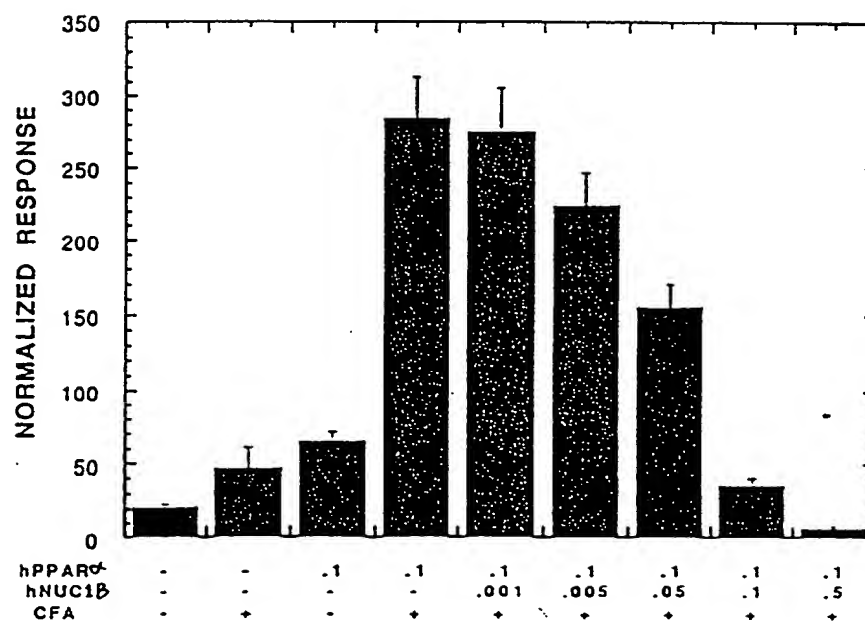
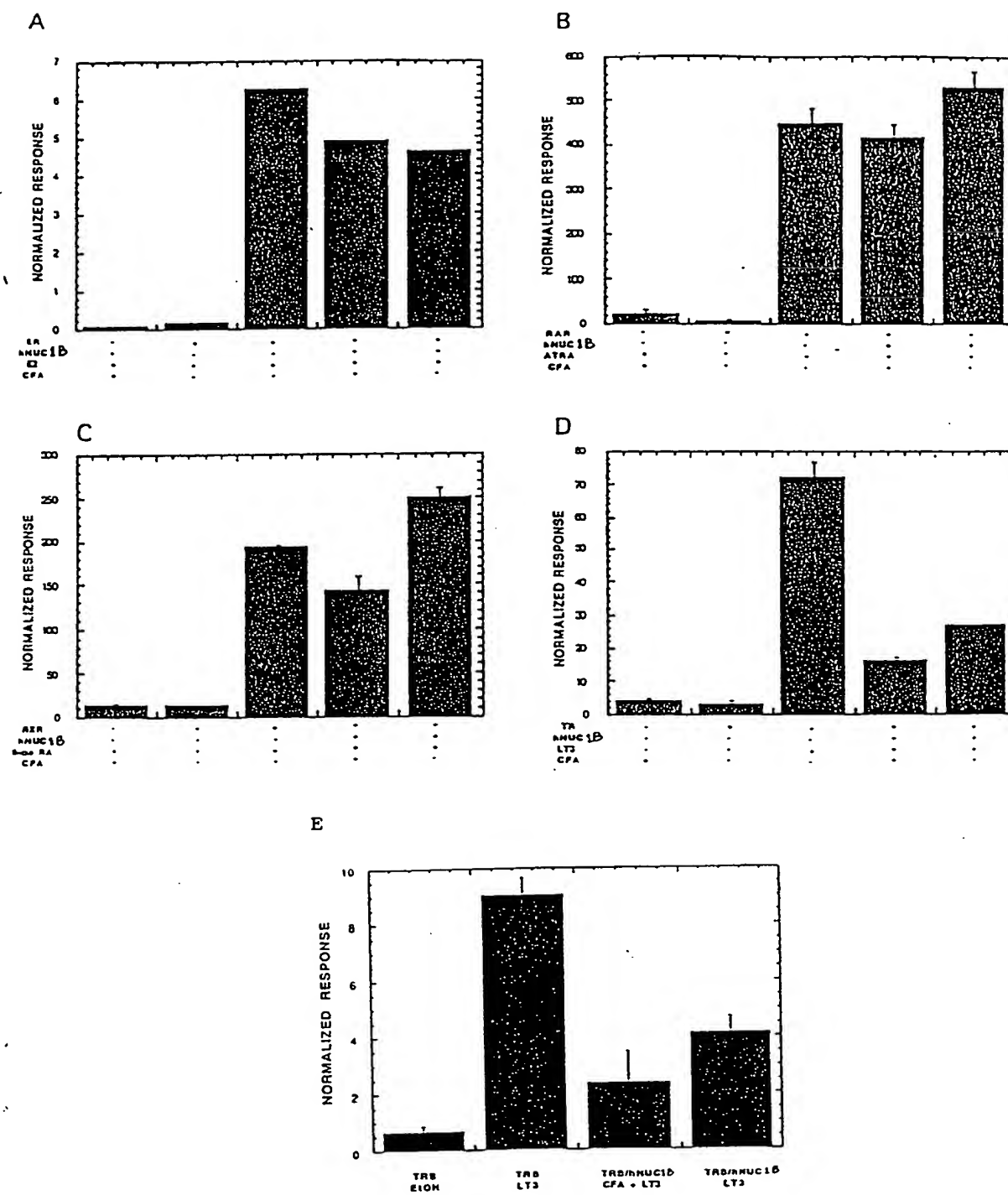
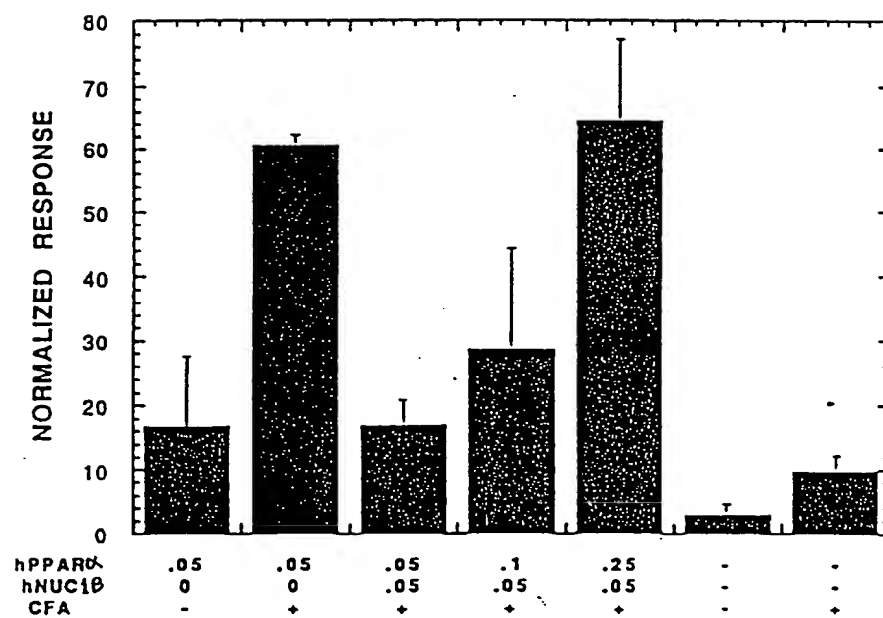


Figure 3



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Figure 4



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Figure 5

hNUC10	+	+	+	-	-
hRXR α	-	+	-	+	+
mock transf.	-	-	+	-	+
	1	2	3	4	5

hRXR α 

1 2 3 4 5

PCT

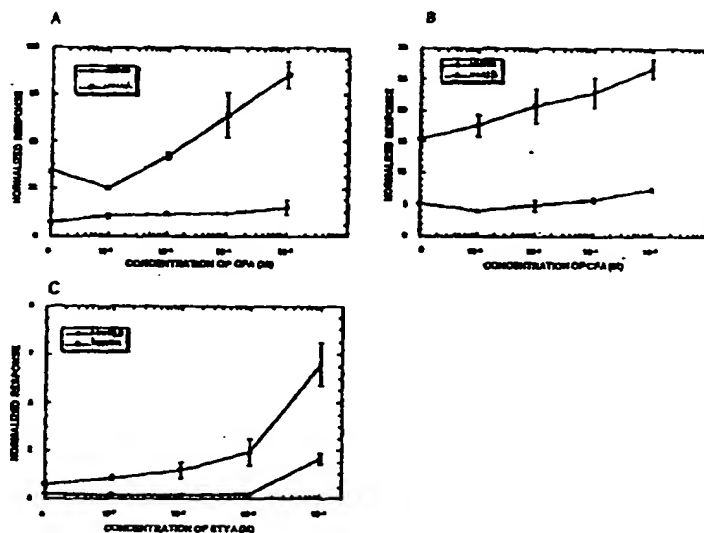
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(74) Agents: WARBURG, Richard, J. et al.; Lyon & Lyon, First Interstate World Center, Suite 4700, 633 West Fifth Street, Los Angeles, CA 90071-2066 (US).			

(54) Title: SCREENING FOR NUC INHIBITORS



(57) Abstract

This invention provides methods for screening for agents useful for treatment of diseases and pathological conditions affected by the level of NUC protein activity. These agents reduce or relieve the repression of PPAR α protein and TR protein transcription activation activity by NUC protein. The selected novel or unique agents can be used to treat hyperlipidemia, hypercholesteremia and hyperlipoproteinemia.

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INTERNATIONAL SEARCH REPORT

International Application No
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A. CLASSIFICATION OF SUBJECT MATTER

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C. DOCUMENTS CONSIDERED TO BE RELEVANT

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A	MOLECULAR ENDOCRINOLOGY, vol. 6, no. 10, 1992, WASHINGTON DC USA, pages 749-784, XP000579262 A. SCHMIDT ET AL.: "Identification of a new member of the steroid hormone receptor superfamily that is activated by a peroxisome proliferator and fatty acids." cited in the application see the whole document ---	1-44
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A,P	WO,A,95 18533 (UNIVERSITY OF PENNSYLVANIA) 13 July 1995 see page 14, line 9 - page 15, line 18 -----	1-44

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International Application No

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